ATTORNEY DOCKET NO. 16016.0005US
Serial No. 08/813,829

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Brigid Hogan, L.M.	)	Group Art Unit: 1632
Serial No. 08/813,829	)	Examiner: Joseph Woitach
Filed: March 6, 1997	)	Confirmation No. 3939
For: STEM CELLS AND METHOD OF MAKING SAME	)	

DECLARATION OF JAMES KELLY UNDER 37 C.F.R. § 1.132

I, James Kelly, a citizen of the United States, residing at 12331 Chesterbrook Dr. Houston, TX 77031 declare that:

1. I have a PhD degree in Biochemistry from Temple University in Philadelphia. I have been conducting research in the field of cell biology and biochemistry since 1975 and am a co-author of at least 50 publications. I am currently president of Amphioxus Cell Technologies, Inc..

2. Amphioxus is currently in negotiation with Plurion, Inc. the licensee of United States Patents 5,670,372, 5,690,926, and 5,453,357, and United States Application No. 08/813,829, (the "'829 application") for the merger/acquisition of Plurion Inc. I may soon have a vested interest in the application for which I am providing this Declaration.

3. I have reviewed the Office Action dated May 18, 2005, as well as the above-identified patents and applications.

4. Based on the following analysis of the '829 application, it is my opinion that the application indicates to someone who understands cell culture and pluripotent stem cell culture and myself that Dr. Hogan had actually isolated human pluripotent stem cells, and she discussed these isolated cells in the application. In addition to my opinion that the application makes clear that Dr. Hogan had isolated human pluripotent cells, experiments were performed which confirm this opinion. While these experiments were not necessary for understanding that Dr. Hogan had isolated human pluripotent stem cells, these experiments prove that that the methods and cells discussed in the '829 application are human pluripotent stem cells.

5. From page 23, line 1 of the '829 application to page 24, line 4, the '829 application provides details about human pluripotent stem cells which were isolated. The methods used were like the methods used for the mouse, further indicating the universal applicability of the methods discussed in the '829 application.

a. The '829 application states: "The above methods for isolation of ES cells from murine embryos were repeated for isolation of ES cells from human embryos. Specifically, testes were dissected from a 10.5 week human embryo. Younger or older embryos represent alternative sources. The preferred age range is between 8.5 weeks and 22 weeks. Tissue was rinsed in buffered saline, and incubated in trypsin solution (0.25% trypsin, 1 mM EDTA in Ca^{30} + /Mg^{++} free HEPES buffered saline) for 10 minutes at 37 C. The tissue was dissociated by pipetting and the cells plated into wells of a 24 well tray containing irradiated feeder cells. In this experiment the feeder cells were Sl/Sl mouse fibroblasts transfected with human membrane associated Stem Cell Factor (Sl^4 h220 cells from Dr. David Williams, HHMI, Indiana State University School of Medicine). An alternative

feeder layer would consist of a mixture of mouse or human embryo fibroblasts and Sl^{4}h220 cells, to provide a more coherent layer for long term cell attachment. The culture medium consists of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum supplemented with 10 ng/ml human bFGF, 60 ng/ml human Stem Cell Factor and 10 ng/ml human LIF. Alternatively, the amounts of bFGF can be increased (e.g. 20 ng/ml). Other alternative or additional supplements can be added at this time, for example IL-6, IL-11, CNTF, NGF, IGFII, flt3/flk2 ligand, and/or members of the Bone Morphogenetic Protein family. The cultures were maintained for 5 days, with daily addition of fresh growth factors. Longer culture could also be utilized, e.g. 5 to 20 days.

b. After 5 days, cultures were dissociated with trypsin solution as before and seeded into wells containing a feeder layer of irradiated mouse embryo fibroblasts. The medium was supplemented with growth factors daily as above. The addition of growth factors to the culture medium at this stage can be utilized, and a feeder layer of a mixture of mouse of human fibroblast and Sl^4 h220 cells can be substituted.

c. After 10 days the cultures were fixed and stained for alkaline phosphatase activity. Colonies of cells expressing high levels of alkaline phosphatase and closely resembling primordial germ cells of the mouse embryo were detected in many wells (see FIG. 5). Closely packed clusters of cells were present in some colonies (arrow in FIG. 5). In cultures of mouse embryo germ cells these colonies give rise to lines of pluripotential embryonic stem cells. Therefore, the identified human cells can give rise to cell lines."

6. It is clear to me from this section of the '829 application that the alkaline phosphatase positive cells were pluripotent stem cells as discussed throughout the '829 application. This is clear because not only did the cells express the alkaline phosphatase, but the cells also resembled the mouse primordial germ cells and the alkaline phosphatase positive cells were in clusters, both characteristics present in the mouse pluripotent stem cells as discussed through out the '829 application. At this point the human pluripotent stem cells had been isolated and were growing and identifiable in the culture.

7. A series of experiments were designed and performed to verify this section in the '829 application. These experiments, described below, replicated the methods described in the '829 application. The original isolation of the cells from the tissue and the first passages were performed by Dr. Rob Hay at the American Type Culture Collection. Frozen vials from the early passage cells were shipped to me and I carried out the remaining experiments. The results paralleled those described in the '829 application. Furthermore, when the cells described in the application as the human pluripotent stem cells were further cultured they behaved with all of the characteristics claimed in the '829 application such as the ability to be passaged at least 20 times and the ability to form embryoid bodies having cells from mesoderm, endoderm, and ectoderm present.

a. A nine week old, male fetus was obtained by Dr. Rob Hay at the American Type Culture Collection. Gonadal ridges were dissected and tissue was dissociated with 0.25% trypsin, 0.03% EDTA. Dissociated cells were plated into two 12.5 cm^2 flasks containing irradiated STO cells as a feeder layer. Cultures were fed with DMEM containing 15% fetal bovine serum (FBS), 1mM glutamine, 0.1 mM nonessential amino acids, 0.1mM beta-mercaptoethanol, 60 ng/ml human stem cell factor, 10 ng/ml human basic

fibroblast growth factor, 10 ng/ml human leukemia inhibitory factor, 100 U/ml penicillin, 100 μg/ml streptomycin. Medium was replaced daily.

b. On day 5, one flask was stained for alkaline phosphatase. There were many positive cells.

c. Cells were passaged on day 6 using 0.05% trypsin, 0.015% EDTA and diluted into four 12.5 cm^2 flasks containing irradiated STO feeder layers. Medium was replaced daily.

d. On day 11, one flask was stained for alkaline phosphatase, showing many positive cells. Cells were trypsinized as above, pooled and plated into two 75 cm^2 flasks and two 12.5 cm^2 flasks containing irradiated STO feeder layers. Medium was replaced daily.

e. On day 20, the cells from the two 75 cm^2 flasks and one 12.5 cm^2 flask were collected by trypsinization, suspended in DMEM containing 15% fetal bovine serum and 10% dimethylsulfoxide and distributed into five 1.5 ml, screw cap freezer vials. Vials were frozen in a controlled rate freezer to a temperature of –80°C, then moved into a liquid nitrogen freezer.

f. Several vials of frozen cells were shipped by Dr. Hay to me. A single vial of cells was thawed and diluted into 10 ml of the DMEM medium described above without the antibiotics. Cells were plated into two wells of a 6 well dish containing mitomycin C treated STO cells. Medium was replaced three times per week. Growth of cells on top of the feeder layer was evident within two days. The cells grew as rounded clusters, evident by their morphology, on top of the flat, fibroblast feeder cells. By day 7,

the medium needed to be changed daily due to pH changes. On day 10, one well was stained for alkaline phosphatase. A nearly confluent monolayer of alkaline phosphatase positive cells was apparent. The other well was trypsinized as described above and the cells were diluted into 15 mls of fresh medium. These were plated into a 75 cm^2 flask containing mitomycin c treated STO cells. Thereafter, cells were trypsinized at approximately ten day intervals and diluted one to five into new flasks containing mitomycin c treated STO cells. This continued for approximately 150 days or 15 passages. At 2.5 doublings per passage, this is a 2^{40} or over a 1 trillion fold expansion of the cells from the frozen vial. Essentially 100% of the cells remain alkaline phosphatase positive.

g. Human pluripotent stem cells were cultured as described above on chambered microscope slides. On day five, cells were washed once with phosphate buffered saline and fixed by treatment with 100% methanol at 4°C for five minutes. Monoclonal antibodies against stage specific embryonic antigen 1 (SSEA1), TRA 1-60 and TRA 1-80 were obtained from Santa Cruz Biotechnology. Antibodies were incubated with the cells at a one to fifty dilution in phosphate buffered saline for two hours at room temperature. Cells were washed three times with PBS then incubated with appropriate second antibodies, either anti mouse IgG or anti mouse IgM. Reaction was detected by staining with an avidin biotin based kit also obtained from Santa Cruz. Cells were positive for all three antigens. Preimmune mouse IgG and IgM were used as controls and showed no reaction.

h. Two 75 cm^2 flasks of human pluripotent cells at approximate passage 21 (the end of the expansion described above) were shipped to Applied Genetics Laboratory in Melbourne, FL, for karyotyping. They found that the cells in the culture, based on 100 metaphases examined, had a modal chromosome number of 46 and 91% of the cells had between 44 and 46 chromosomes. No chromosome aberrations, defined as deletions, inversions or interchanges of genetic materials, were found in the 100 metaphase cells examined. This is a normal karyotype.

i. Human pluripotent stem cells were trypsinized as described above and resuspended in Med3, which is a mixture of Ham's F12, Williams E and Waymouth's MAB containing 5% defined calf serum. The cell suspension was placed into a non adherent plastic container and incubated without medium change for five days. Within twenty four hours, most of the cells had formed aggregates of cells. In an additional 24 hours, most of the aggregates had formed hollow centered balls of cells, so called embryoid bodies. At five days, half of the medium was removed and replaced. Aggregates began to show signs of developing an external layer of differentiated cells with a different morphology than the internal cells. Aggregates expanded considerably in size.

j. An aliquot of the medium containing the embryoid bodies was removed and cells were collected by centrifugation. Cells were suspended in fresh Med3 and plated in the wells of a 6 well tissue culture dish. Most aggregates adhered to the surface of the wells and cell proliferation was evident. Within ten days, most wells contained nearly confluent layers of

cells with a significantly different morphology than the untreated pluripotent stem cells.

8. Thus, the '829 application not only taught how to make human pluripotent stem cells, the '829 application also discusses a completed isolation of human pluripotent stem cells. The cells were in the hands of the Inventor, listed as Dr. Brigid Hogan, based on what is disclosed in the '829 application.

9. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or document or any patent issuing therefrom.

James H Kelly
James Kelly, Ph.D.

18 November 2005
DATE